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(57) Abstract

An assay, reagents and a test kit useful for detecting the presence of anti-HEV antibody which may be present in a test sample. The assay method comprises (a) contacting the test sample with at least one synthetic peptide which specifically binds anti-HEV antibody, for a time and under conditions sufficient to form peptide/antibody complexes; (b) contacting said peptide/antibody complexes with an indicator reagent which comprises a signal generating compound attached to an anti-human antibody, and incubating for a time and under conditions sufficient for peptide/antibody/antibody complexes to form; and (c) detecting a measurable signal to indicate the presence of anti-HEV in the test sample.

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CONFIRMATORY ASSAY AND REAGENTS FOR HEPATITIS E VIRUS

Background of the Invention

This invention relates generally to Hepatitis E Virus (HEV), and more particularly, relates to assays useful for confirming the presence of antibodies to HEV in test samples.

HEV, variously referred to as waterborne, epidemic or enterically transmitted non-A, non-B hepatitis (ET-NANBH) has a global distribution and has been noted as the cause of major endemic outbreaks of viral hepatitis in developing countries. D. W. Bradley et al., Br. Med. Bull. 46:442-461 (1990). Sporadic cases of ET-NANBH, as well as imported travel exposure, have been reported in developed countries. S. J. Skidmore et al., Lancet 337:1541 (1991). Although the fecal-oral route of transmission predominates, limited person-to-person routes of exposure have been suggested in some epidemiologic studies. O. Velasquez et al., J. Amer. Med. Assoc. 363:3281-3285 (1990). This disease has been documented as having a high mortality rate of approximately 20% in pregnant women infected during their third trimester of pregnancy. See D. W. Bradley et al., supra.

Molecular cloning of the putative agent of HEV has been hampered by the lack of a tissue culture system for virus propagation. However, the use of available animal models and a newly developed non-specific amplification procedure have allowed the identification of a unique cDNA clone (identified as "ET 1.1") obtained from bile of cynomolgus macaques infected with a Burmese strain of HEV. A. G. Andjaparidze et al., Vopr. Virusol. 1:73-80 (1986), D. W. Bradley et al., Proc. Natl. Acad. Sci. USA 84:6277-6281 (1987) and G. W. Reyes et al., Science 247:1335-1339 (1990). Successful confirmation of the viral origin of this clone led to the identification of similar sequences in human fecal specimens collected from ET-NANBH epidemics in Somalia, Tashkent, Borneo, Pakistan and Mexico. See G. R. Reyes et al., supra. cDNA libraries also have been prepared from human stool samples obtained during an ET-NANBH outbreak in Mexico. G. R. Reyes et al., Gastroenterol. Japon. 26:142-147 (1991). Immunoscreening of these cDNA libraries led to the identification of two cDNA clones which encode epitopes specific for HEV. P. O. Yarbough et al., J. Virol. 65:5790-5797 (1991). The isolation and sequencing of a set of overlapping cDNA clones led to the recognition that this form of hepatitis is caused by a novel virus unlike any of the other molecularly characterized agents of viral hepatitis. A. W. Tam et al., Virology 185:12-131 (1991).

Various regions of the HEV genome have been cloned and expressed in E. coli as fusion proteins with glutathione-S-transferase (GST). See, for example, S. J. Skidmore et al., supra. Four of these recombinant antigens, two derived from a Burmese (B) strain of HEV and two derived from a Mexican (M) strain of HEV, have been shown to contain antigenic sites recognized by antibodies from individuals previously exposed to HEV. See, P. O. Yarbough et al., supra. The two antigens from the Mexican strain, named M 3-2 and M 4-2, correspond to amino acid sequences at the carboxy-terminus of the second open reading frame (ORF-2) and the third open reading frame (ORF-3), respectively. The two antigens from the Burmese strain, B 3-2 and B 4-2, correspond to amino acid sequences at the carboxy-terminus of ORF-2 and ORF-3, respectively. The M 3-2 and B 3-2 recombinant antigens both comprise 42 amino acids from the carboxy terminus of ORF-2. The degree of amino acid homology between these sequences of 42 amino acids is 90.5%. Id. The M 4-2 and B 4-2 recombinant antigens each comprise 33 amino acids from the carboxy terminus of ORF-3; the degree of homology between these two sequences of 33 amino acids is 73.5%. Id.

The M 3-2, M 4-2, B 3-2 and B4-2 recombinant antigens have been used in assays designed to determine the presence of HEV antibody in an individual by assaying the individual's test sample for anti-HEV antibody. The use of recombinant technology, however, may lead to false positive results, which may occur for two major reasons. The first reason is that the process of producing the desired recombinant antigens involves producing these antigens in microbiological cultures usually of a bacterial or yeast source. During this process, the bacterial or yeast protein co-purifies with the recombinant antigen. Consequently, when the purified recombinant antigen is utilized in an assay in the capture phase, the co-purified bacterial or yeast protein also is present. A test sample may have antibodies to either or both the recombinant antigen and the co-purified bacterial protein. When a test sample having antibodies to the co-purified protein is reacted with such a recombinant antigen used to capture antibody to the analyte of interest in the test sample, the antibodies in the test sample bind to the co-purified protein. False positive reactions thus may occur when the test sample reacts with the co-purified bacterial or yeast protein and falsely yields a "positive" reaction for the analyte of interest. Second, recombinant HEV antigens are expressed as fusion proteins in GST, and it is known that some individuals produce antibody to GST. Thus, a false positive reaction also can occur in assays employing HEV recombinant antigens which have been cloned and expressed in E. coli as fusion proteins with GST, since an individual may react only to GST yet demonstrate a

positive reaction in an assay for antibody to an HEV specific analyte, for which analyte the recombinant protein was being used as a capture antigen. Further, special manufacturing facilities may be required for producing the large amounts of recombinant antigens required for commercial use, and the manufacture of recombinant antigens within the stringent regulatory paramenters necessary for their commercial application may be problematic at times.

While methods are available to confirm the presence of screening assay results for agents such as HIV, these techniques are not yet available for confirming the presence of HEV. Thus, methods such as culturing HEV in vitro and the Western blot test are not available. While the detection of HEV nucleic acid may be attempted by performing PCR, this technique is tedious and expensive, requires special equipment such as a thermocycler, and turn-around time is up to 24 hours. Immunoelectron microscopy (IEM) has been used to confirm the presence of anti-HEV antibody, but the use of IEM is cost prohibitive as a routine confirmatory tool.

It therefore would be advantageous to provide a method for confirming the presence of HEV antibody in a test sample that is accurate, rapid and cost effective.

Summary of the Invention

The present invention provides an assay for confirming the presence of anti-HEV antibody in a test sample, which assay comprises: (a) contacting the test sample with at least one synthetic peptide which specifically binds anti-HEV antibody, for a time and under conditions sufficient to form peptide/antibody complexes; (b) contacting said peptide/antibody complexes with an indicator reagent which comprises a signal generating compound attached to an anti-human antibody, and incubating for a time and under conditions sufficient for peptide/antibody/antibody complexes to form; and (c) detecting a measurable signal to indicate the presence of anti-HEV in the test sample.

The amino acid sequences of the HEV synthetic peptides used in the present invention were obtained from the predicted amino acid sequences as published by P. O. Yarbough et al., J. Virol. 65: 5790-5797 (1991). These synthetic peptides include: SPM 42 (SEQ. ID. NO. 1, corresponding to the recombinant antigen M 4-2), SPB 42 (SEQ. ID. NO. 2, corresponding to the recombinant antigen B4-2), SPB 33 (SEQ. ID. NO. 3, corresponding to the recombinant antigen B 3-2), and SPM 33 (SEQ. ID. NO. 4, corresponding to the recombinant antigen M 3-2).

The present invention also provides kits containing at least one synthetic HEV peptide of the invention useful for assaying a test sample for the presence of anti-HEV antibody.

Detailed Description of the Invention

The present invention provides an assay, synthetic peptides and a kit useful for detecting antibodies to Hepatitis E Virus (HEV). Since the sequences of the present invention are manufactured by synthetic processes, they do not have the problems associated with recombinant proteins prepared in GST, nor do they not contain extraneous bacterial or yeast proteins which often co-purify with the recombinant antigen. The synthetic peptides disclosed herein thus provide advantages over the recombinant antigens known in the art by avoiding the cross reactivity problems from which these recombinant proteins suffer.

In addition to their use in various assays, the synthetic peptides can be bound to matrices similar to CNBr-activated sepharose and used for the affinity purification of antibodies specific for HEV antigens from cell cultures, or biological tissues such as blood and liver. Further, the synthetic peptides of the invention can be used for the generation of chimeric, monoclonal or polyclonal antibodies for therapeutic use, or similar applications.

The synthetic peptides of the present invention provided herein may be used singly or in combination. For example, one or more peptides of the invention may be attached to a single solid phase for use in various assay systems. Or, each individual peptide may be attached to a solid phase such as a microparticle and thereafter combined with other so-prepared microparticles for use in an assay. Assays then cAN be optimized by varying the peptides and/or concentration of peptides in the assay. Methods for optimizing assays are known to those of ordinary skill in the art. Several methods for attachment are known in the art and include coating and covalent linking.

"Test samples" which can be tested by the methods of the present invention described herein include human or animal biological fluids such as whole blood, serum, plasma, cerebral spinal fluid, urine, ascites or any other body constituents or any tissue culture supernatants which might contain the antibodies of interest.

A "solid phase", as used herein, refers to any material which is insoluble, or can be made insoluble by a subsequent reaction. The solid phase can be chosen for its intrinsic ability to attract and immobilize the capture reagent. Alternatively, the solid phase can retain an additional receptor which has the ability to attract and immobilize the capture reagent. The additional receptor can include a charged

substance that is oppositely charged with respect to the capture reagent itself or to a charged substance conjugated to the capture reagent. As yet another alternative, the receptor molecule can be any specific binding member which is immobilized upon (attached to) the solid phase and which has the ability to immobilize the capture reagent through a specific binding reaction. The receptor molecule enables the indirect binding of the capture reagent to a solid phase material before the performance of the assay or during the performance of the assay. The solid phase thus can be a plastic, derivatized plastic, magnetic or non-magnetic metal, glass or silicon surface of a test tube, microtiter well, sheet, bead, microparticle, chip, and other configurations known to those of ordinary skill in the art.

It is contemplated and within the scope of the invention that the solid phase also can comprise any suitable porous material with sufficient porosity to allow access by detection antibodies and a suitable surface affinity to bind antigens. Microporous structures are generally preferred, but materials with gel structure in the hydrated state may be used as well. Such useful solid supports include: natural polymeric carbohydrates and their synthetically modified, crosslinked or substituted derivatives, such as agar, agarose, cross-linked alginic acid, substituted and cross-linked guar gums, cellulose esters, especially with nitric acid and carboxylic acids, mixed cellulose esters, and cellulose ethers; natural polymers containing nitrogen, such as proteins and derivatives, including cross-linked or modified gelatins; natural hydrocarbon polymers, such as latex and rubber; synthetic polymers which may be prepared with suitably porous structures, such as vinyl polymers, including polyethylene, polypropylene, polystyrene, polyvinylchloride, polyvinylacetate and its partially hydrolyzed derivatives, polyacrylamides, polymethacrylates, copolymers and terpolymers of the above polycondensates, such as polyesters, polyamides, and other polymers, such as polyurethanes or polyepoxides; porous inorganic materials such as sulfates or carbonates of alkaline earth metals and magnesium, including barium sulfate, calcium sulfate, calcium carbonate, silicates of alkali and alkaline earth metals, aluminum and magnesium; and aluminum or silicon oxides or hydrates, such as clays, alumina, talc, kaolin, zeolite, silica gel, or glass (these materials may be used as filters with the above polymeric materials); and mixtures or copolymers of the above classes, such as graft copolymers obtained by initializing polymerization of synthetic polymers on a pre-existing natural polymer. All of these materials may be used in suitable shapes, such as films, sheets, or plates, or they may be coated onto or bonded or laminated to appropriate inert carriers, such as paper, glass, plastic films, or fabrics.

The porous structure of nitrocellulose has excellent absorption and adsorption qualities for a wide variety of reagents including monoclonal antibodies. Nylon also possesses similar characteristics and also is suitable.

It is contemplated that such porous solid supports described hereinabove are preferably in the form of sheets of thickness from about 0.01 to 0.5 mm, preferably about 0.1 mm. The pore size may vary within wide limits, and is preferably from about 0.025 to 15 microns, especially from about 0.15 to 15 microns. The surfaces of such supports may be activated by chemical processes which cause covalent linkage of the antigen or antibody to the support. The irreversible binding of the antigen or antibody is obtained, however, in general, by adsorption on the porous material by poorly understood hydrophobic forces.

Preferred solid phase materials for flow-through assay devices include filter paper such as a porous fiberglass material or other fiber matrix materials. The thickness of such material is not critical and will be a matter of choice, largely based upon the properties of the sample or analyte being assayed, such as the fluidity of the test sample.

To change or enhance the intrinsic charge of the solid phase, a charged substance can be coated directly to the material or onto microparticles which then are retained by a solid phase support material. Alternatively, microparticles can serve as the solid phase, by being retained in a column or being suspended in the mixture of soluble reagents and test sample, or the particles themselves can be retained and immobilized by a solid phase support material. By "retained and immobilized" is meant that the particles on or in the support material are not capable of substantial movement to positions elsewhere within the support material. The particles can be selected by one skilled in the art from any suitable type of particulate material and include those composed of polystyrene, polymethylacrylate, polypropylene, latex, polytetrafluoroethylene, polyacrylonitrile, polycarbonate, or similar materials. The size of the particles is not critical, although it is preferred that the average diameter of the particles be smaller than the average pore size of the support material being used. Thus, embodiments which utilize various other solid phases also are contemplated and are within the scope of this invention. For example, ion capture procedures for immobilizing an immobilizable reaction complex with a negatively charged polymer, described in co-pending U. S. Patent Application Serial No. 150,278 corresponding to EP Publication No. 0326100, and U. S. Patent Application Serial No. 375,029 (EP Publication No. 0406473), can be employed according to the present invention to effect a fast solution-phase immunochemical reaction. An

immobilizable immune complex is separated from the rest of the reaction mixture by ionic interactions between the negatively charged polyanion/immune complex and the previously treated, positively charged porous matrix and detected by using various signal generating systems previously described, including those described in chemiluminescent signal measurements as described in co-pending U.S. Patent Application Serial No. 921,979 corresponding to EPO Publication No. 0 273,115.

Also, the methods of the present invention can be adapted for use in systems which utilize microparticle technology including automated and semi-automated systems wherein the solid phase comprises a microparticle. Such systems include those described in pending U. S. Patent Application 425,651 and U. S. Patent No. 5,089,424, which correspond to published EPO applications Nos. EP O 425 633 and EP 0 424 634, respectively, and U.S. Patent No. 5,006,309.

The "indicator reagent" may comprise a signal generating compound (label) which is capable of generating a measurable signal detectable by external means conjugated (attached) to a specific binding member for anti-HEV antibody. "Specific binding member," as used herein, means a member of a specific binding pair. That is, two different molecules where one of the molecules through chemical or physical means specifically binds to the second molecule. In addition to being an antibody or antibody fragment member of a specific binding pair for anti-HEV antibody, the indicator reagent also can be a member of any specific binding pair, including either hapten-anti-hapten systems such as biotin or antibiotin, avidin or biotin, a carbohydrate or a lectin, a complementary nucleotide sequence, an effector or a receptor molecule, an enzyme cofactor and an enzyme, an enzyme inhibitor or an enzyme, and the like.

The various signal generating compounds (labels) contemplated include chromogens; catalysts such as enzymes, for example, alkaline phosphatase, horseradish peroxidase, beta-galactosidase, and the like; luminescent compounds such as fluorescein and rhodamine, chemiluminescent compounds such as acridinium, phenanthridinium, dioxetanes, luminol, and the like; radioactive elements; and direct visual labels. The selection of a particular label is not critical, but it will be capable of producing a signal either by itself or in conjunction with one or more additional substances.

The synthetic peptides of the present invention can be employed in various assay systems to confirm the presence of anti-HEV antibody in a test sample that previously tested reactive (or "positive") for the presence of anti-HEV antibody. These peptides are identified as SEQ. ID. NO. 1 corresponding to recombinant

antigen M 4-2 , SEQ. ID. NO. 2 corresponding to recombinant antigen B 4-2, SEQ. ID. NO. 3 corresponding to recombinant antigen B 3-2 and SEQ. ID. NO. 4 corresponding to recombinant antigen M 3-2. In a first embodiment, the present invention provides an assay which comprises the steps of (a) contacting a test sample with at least one HEV synthetic peptide and incubating the so-formed mixture for a time and under conditions sufficient to form peptide/antibody complexes; (b) contacting the peptide/antibody complexes with an indicator reagent which comprises an anti-human antibody or a fragment thereof attached to a signal generating compound capable of generating a detectable measurable signal, and incubating this second so-formed mixture for a time and under conditions sufficient to form peptide/antibody/indicator reagent complexes; and (c) detecting the measurable signal generated by the signal generating compound as an indication of the presence of anti-HEV antibody in the test sample. Preferably, the capture peptide is attached to a solid phase prior to its use in the assay. If a solid phase is used, it can be separated from the liquid phase prior to the detection of the signal generating compound. Moreover, steps (1) and (2) can be performed simultaneously. It also is contemplated and within the scope of the invention that the test sample can be diluted in all of the assay embodiments, and that washing occurs or can occur between steps of all assay formats described herein.

Alternatively, a test sample previously reactive for anti-HEV antibody is contacted with a solution phase capture reagent which comprises an HEV antigen (sequence of amino acids coded by HEV which induce an immune response [e.g. antibody production]) which specifically binds anti-HEV antibody, and incubated for a time and under conditions sufficient for antibody/antigen complexes to form. Next, the antibody/antigen complexes are contacted with a solid phase capture reagent which comprises a synthetic HEV peptide containing amino acids found within the HEV antigen used in the first step. The complex and solid phase are incubated for a time and under conditions sufficient for peptide/antibody complexes to form. Then the solid phase is separated from the solution and contacted with an indicator reagent comprising a monoclonal or a polyclonal anti-human antibody or a fragment thereof which has been attached to a signal generating compound capable of generating a measurable signal, to form a mixture. This mixture is incubated for a time and under conditions sufficient to form peptide/antibody/indicator reagent complexes. The presence of immobilized antibody is determined by detecting the measurable signal generated. A decrease in the amount of signal generated, as compared to an initial screening, confirms the presence of anti-HEV antibody present in the test sample.

In another embodiment, at least one of the HEV synthetic peptide is immobilized on a nitrocellulose membrane. The peptide also can be conjugated or crosslinked to itself, other peptides or to various carrier proteins such as BSA, keyhole limpet hemocyanin, ovalbumin, and the like, before immobilization on the nitrocellulose membrane. The test sample is incubated on the membrane for a time and under conditions sufficient for peptide/antibody complexes to form. After removing unbound proteins, the membrane is incubated with an indicator reagent comprising anti-human antibodies labelled with a signal generating compound. The presence and/or amount of anti-HEV antibody present in the test sample is determined by detecting the measurable signal. The amount of signal is proportional to the amount of anti-HEV present in a test sample.

Other embodiments which utilize various other solid phases also are contemplated and are within the scope of this invention. For example, ion capture procedures for immobilizing an immobilizable reaction complex with a negatively charged polymer, described in co-pending U. S. Patent Application Serial No. 150,278 corresponding to EP publication 0 326 100, and U. S. Patent Application Serial No. 375,029 (EP publication No. 0 406 473) both of which enjoy common ownership and are incorporated herein by reference, can be employed according to the present invention to effect a fast solution-phase immunochemical reaction. An immobilizable immune complex is separated from the rest of the reaction mixture by ionic interactions between the negatively charged poly-anion/immune complex and the previously treated, positively charged porous matrix and detected by using various signal generating systems previously described, including those described in chemiluminescent signal measurements as described in co-pending U.S. Patent Application Serial No.921,979 corresponding to EPO Publication No. 0 273 115, which enjoys common ownership and which is incorporated herein by reference.

Also, the methods of the present invention can be adapted for use in systems which utilize microparticle technology including automated and semi-automated systems wherein the solid phase comprises a microparticle. Such systems include those described in pending U.S. Patent Application Serial Nos. 426,651 and 426,643, which correspond to published EPO applications Nos. EP 0 425 633 and EP 0 424 634, respectively, which are incorporated herein by reference.

The use of scanning probe microscopy (SPM) for immunoassays also is a technology to which the synthetic peptides of the present invention are easily adaptable. In scanning probe microscopy, in particular in atomic force microscopy, the capture phase, for example, at least one of the synthetic peptides

of the invention, is adhered to a solid phase and a scanning probe microscope is utilized to detect antigen/antibody complexes which may be present on the surface of the solid phase. The use of scanning tunnelling microscopy eliminates the need for labels which normally must be utilized in many immunoassay systems to detect antigen/antibody complexes. Such a system is described in pending U.S. Patent Application Serial No. 662,147, which enjoys common ownership and is incorporated herein by reference.

The use of SPM to monitor specific binding reactions can occur in many ways. In one embodiment, one member of a specific binding partner (antibody specific substance which is the synthetic peptide of the invention) is attached to a surface suitable for scanning. The attachment of the antibody specific substance may be by adsorption to a test piece which comprises a solid phase of a plastic or metal surface, following methods known to those of ordinary skill in the art. Or, covalent attachment of a specific binding partner (antibody specific substance) to a test piece which test piece comprises a solid phase of derivatized plastic, metal, silicon, or glass may be utilized. Covalent attachment methods are known to those skilled in the art and include a variety of means to irreversibly link specific binding partners to the test piece. If the test piece is silicon or glass, the surface must be activated prior to attaching the specific binding partner. Activated silane compounds such as triethoxy amino propyl silane (available from Sigma Chemical Co., St. Louis, MO), triethoxy vinyl silane (Aldrich Chemical Co., Milwaukee, WI), and (3-mercapto-propyl)-trimethoxy silane (Sigma Chemical Co., St. Louis, MO) can be used to introduce reactive groups such as amino-, vinyl, and thiol, respectively. Such activated surfaces can be used to link the binding partner directly (in the cases of amino or thiol) or the activated surface can be further reacted with linkers such as glutaraldehyde, bis (succinimidyl) suberate, SPPD 9 succinimidyl 3-[2-pyridyldithio] propionate), SMCC (succinimidyl-4-[Nmaleimidomethyl] cyclohexane-1-carboxylate), SIAB (succinimidyl [4iodoacetyl] aminobenzoate), and SMPB (succinimidyl 4-[1maleimidophenyl] butyrate) to separate the binding partner from the surface. The vinyl group can be oxidized to provide a means for covalent attachment. It also can be used as an anchor for the polymerization of various polymers such as poly acrylic acid, which can provide multiple attachment points for specific binding partners. The amino surface can be reacted with oxidized dextrans of various molecular weights to provide hydrophilic linkers of different size and capacity. Examples of oxidizable dextrans include Dextran T-40 (molecular weight 40,000 daltons), Dextran T-110 (molecular weight 110,000 daltons), Dextran T-500 (molecular weight 500,000

daltons), Dextran T-2M (molecular weight 2,000,000 daltons) (all of which are available from Pharmacia, Piscataway, NJ), or Ficoll (molecular weight 70,000 daltons (available from Sigma Chemical Co., St. Louis, MO). Also, polyelectrolyte interactions may be used to immobilize a specific binding partner on a surface of a test piece by using techniques and chemistries described by pending U. S. Patent applications Serial No. 150,278, filed January 29, 1988, and Serial No. 375,029, filed July 7, 1989, each of which enjoys common ownership and each of which is incorporated herein by reference. The preferred method of attachment is by covalent means. Following attachment of a specific binding member, the surface may be further treated with materials such as serum, proteins, or other blocking agents to minimize non-specific binding. The surface also may be scanned either at the site of manufacture or point of use to verify its suitability for assay purposes. The scanning process is not anticipated to alter the specific binding properties of the test piece.

While the present invention discloses the preference for the use of solid phases, it is contemplated that the peptides of the present invention can be utilized in non-solid phase assay systems. These assay systems are known to those skilled in the art, and are considered to be within the scope of the present invention.

It is contemplated that the reagent employed for the assay can be provided in the form of a kit with one or more containers such as vials or bottles, with each container containing a separate reagent such as a peptides, or a mixture of peptides, employed in the assay. These kits also could contain vials or containers of other reagents needed for performing the assay, such as washing, processing and indicator reagents.

The present invention will now be described by way of examples, which are meant to illustrate, but not to limit, the scope of the present invention.

Example 1

Synthetic of Synthetic Peptides

A. Synthesis of SPB 42 (SEQ. ID. NO. 2)

The fully protected peptide-resin was assembled on a phenylacetamidomethyl (PAM) resin by stepwise solid phase synthesis (starting with the carboxyl terminal residue) according to the procedure of G. Barany and R. B. Merrifield, The Peptides, (E. Gross and T Meinhoeffer, eds) 2, 1-284 (1980), Academic Press, New York, N.Y, as follows. The C-terminal amino acid arginine (Arg) was coupled to the solid support via an

oxymethylphenylacetamidomethyl (OMPA) linkage to yield PAM resin which ensures improved stability to prolonged treatment with trifluoroacetic acid (TFA). A BOC-Arg(TOS)-OCH2-PAM-resin (0.58 mmoVg, 0.16148 g) was transferred to the reaction vessel of an Applied Biosystems, Inc. (ABI) Peptide Synthesizer, model 430A (available from ABI, Foster City, CA). All subsequent amino acids starting from the carboxyl terminal to N-terminus were coupled in a stepwise manner using ABI's 0.1 mmol small scale rapid cycle protocol with capping (available from ABI, Product Number [P/N] 601781, version 1.40). Protected amino acids were coupled using preformed symmetric anhydride chemistry except for asparagine, glutamine, arginine and histidine which were double coupled using N-N'-dicyclohexylcarbodiimide (DCC)/1-hydroxybenzotriazole (HOBT) chemistry. In the first coupling, protected amino acids were coupled using acetic anhydride dissolved in dimethylformamide (DMF). The acetic anhydride of an individual amino acid was formed in methylene chloride followed by solvent exchange to DMF before transferring to the reaction vessel of the peptide synthesizer. The second coupling of symmetric anhydride also was conducted in DMF. The N-amino group of all amino acids used was protected by a t-butyloxycarbonyl (t-BOC) linkage. The side chain functional groups of various amino acids were protected by the following groups:

Ser-Bzl	(Benzyl)
Asp,Glu-OBzl	(O-Benzyl)
His-DNP	(Dinitrophenol)
Arg-Tos	(Tosylate)

The fully protected peptide-resin (0.338g) was allowed to swell in methylene chloride (CH_2Cl_2) for five minutes. The peptide-resin was transferred to a manual reaction vessel, treated twice with 5% thiophenol in DMF for twenty minutes each followed by six CH_2Cl_2 washes for one minute each, and then transferred to the reaction vessel of the synthesizer. The t-BOC protecting group was then removed using 60% TFA in CH_2Cl_2 according to the manufacturer's protocol (available from ABI, Foster City. CA) and the partially deprotected peptide-resin was then dried overnight under house vacuum at room temperature.

Partially deprotected peptide-resin was then treated with p-cresol (1 ml) in anhydrous hydrogen fluoride (HF, 9 ml) at 0°C for two hours to cleave the peptide from the resin support. The HF/DMS and other volatiles were distilled off in vacuo at 0°C. The cleaved peptide and resin were washed three times with 15 ml aliquots of diethyl ether, and the cleaved peptide was extracted by washing three times each with 10 ml aliquots of 40% aqueous acetic acid and 16% aqueous acetic

acid, respectively. The aqueous extracts were combined and washed three times with 15 ml aliquots of diethyl ether and then lyophilized to yield a crude peptide.

The crude peptide was analyzed for purity using reversed-phase high performance liquid chromatography on a C4, 4.6 x 30 mm column (available from Vydac, The Separations Group, Hesperia, CA), flow rate one ml/minute employing 0.1% aqueous TFA (A) and 100% acetonitrile (B) as the solvent system. The preferred solvent gradient employed for this peptide analysis started with 20% B solvent. The column was maintained at 20% B for five minutes followed by an increase over 20 minutes using a linear gradient to 50% B and maintained for one minute. Finally, the column was brought back to 20% B over a two minute period. The presence of peptide in the effluent was monitored simultaneously at 225 nm and 254 nm. The composition of the purified peptide was determined by acid hydrolysis. After removal of the acid, the hydrosylate was analyzed on a Beckman 6300 amino acid analyzer.

If increased quantities of purified polypeptide were desired, semipreparative reversed phase high performance liquid chromatography was performed in a similar manner using a C18, 10 x 100 mm column (Vydac, The Separations Group, Hesperia, California) using the same aqueous 0.1% TFA (A) and 100% acetonitrile (B) solvent system described above. The preferred solvent gradient for a semi-preparative run started with 21% B at 3 ml/minute for five minutes followed by an increase, over 20 minutes, using a linear gradient to 40% B. The concentration was maintained at 40% B for one minute and then reduced to 21% B in one minute.

B. Synthesis of SPB 33 (SEQ. ID. NO. 3)

The fully protected peptide-resin was assembled on a phenylacetamidomethyl (PAM) resin by stepwise solid phase synthesis (starting with the carboxyl terminal residue) according to the general procedure of Barany and Merrifield, Id. The C-terminal amino acid arginine (Arg) was coupled to the solid support vial an oxymethylphenylacetamidomethyl (OMPA) linkage to yield PAM resin which ensures improved stability to prolonged treatment with trifluoroacetic acid (TFA). A BOC-Arg(TOS)-OCH_2-PAM-resin (0.58 mmoVg, 0.17195 g) was transferred to the reaction vessel of an ABI Peptide Synthesizer, model 430A. All subsequent amino acids starting from the carboxyl terminal to N-terminus were coupled in a stepwise manner using ABI's 0.1 mmol small scale rapid cycle protocol with capping (available from ABI, Froster City, CA, P/N 601781, version 1.40). Protected amino acids were coupled using preformed

symmetric anhydride chemistry except for asparagine, glutamine, arginine and histidine which were double coupled using N-N'-dicyclohexylcarbodiimide (DCC)/1-hydroxybenzotriazole (HOBT) chemistry. In the first coupling, protected amino acids were coupled using acetic anhydride dissolved in dimethylformamide (DMF). The acetic anhydride of an individual amino acid was formed in methylene chloride followed by solvent exchange to DMF before transferring to the reaction vessel of the peptide synthesizer. The second coupling of symmetric anhydride was also conducted in DMF. The N-amino group of all amino acids used was protected by a t-butyloxycarbonyl (t-BOC) linkage. The side chain functional groups of various amino acids were protected by the following groups:

Thr, Ser-Bzl	(Benzyl)
Asp-Ochxl	(O-Cyclohexyl)
His-pBom	(p-Benzyloxy methyl)
Arg-Tos	(Tosyl)

Prior to placing the resin in the reaction vessel of the synthesizer, the fully protected peptide-resin (0.25527g) was allowed to swell in methylene chloride (CH_2Cl_2) for five minutes. The t-BOC protecting group then was removed using 60% TFA/CH_2Cl_2 according to the manufacturer's protocol and the partially deprotected peptide-resin was then dried overnight under house vacuum at room temperature.

Partially deprotected peptide-resin then was treated with p-cresol (1 ml) in anhydrous HF (9 ml) at 0°C for one and three quarters hours to cleave the peptide from the resin support. The HF/DMS and other volatiles were distilled off in vacuo at 0°C. The cleaved peptide and resin were washed three times with 15 ml aliquots of diethyl ether, and the cleaved peptide was extracted by washing three times each with 10 ml aliquots of 40% aqueous acetic acid and 15% aqueous acetic acid, respectively. The aqueous extracts were combined and washed three times with 15 ml aliquots of diethyl ether and then lyophilized to yield a crude peptide.

The crude peptide was analyzed for purity using reversed-phase high performance liquid chromatography on a C4, 4.6 x 30 mm column (Brownlee, available from ABI, Foster City, CA), flow rate one ml/minute employing 0.1% aqueous TFA (A) and 100% acetonitrile (B) as the solvent system. The preferred solvent gradient employed for this peptide analysis started with 12% B solvent. The column was maintained at 12% B for one minute followed by an increase over 20 minutes using a linear gradient to 29% B and maintained for one minute. Finally, the column was brought back to 12% B in one minute. The presence of

peptide in the effluent was monitored simultaneously at 226 nm and 264 nm. The composition of the purified peptide was determined by acid hydrolysis. After removal of the acid, the hydrosylate was analyzed on a Beckman 6300 amino acid analyzer.

If increased quantities of purified polypeptide were desired, semipreparative reversed phase high performance liquid chromatography was performed in a similar manner using a C4, 10 x 100 mm column (available from Vydac, The Separations Group, Hesperia, CA) using the same aqueous 0.1% TFA (A) and 100% acetonitrile (B) solvent system described above. The preferred solvent gradient for a semi-preparative run started with 17% B at 3 ml/minute for five minutes followed by an increase, over 20 minutes, using a linear gradient to 28% B. The concentration was maintained at 28% B for one minute and then returned to 17% B in one minute.

C. Synthesis of SPM 33 (SEQ. ID. NO. 4)

The fully protected peptide-resin was assembled on a phenylacetamidomethyl (PAM) resin by stepwise solid phase synthesis (starting with the carboxyl terminal residue) according to the general procedure of Barany and Merrifield mentioned previously. The C-terminal amino acid valine (Val) was coupled to the solid support vial an oxymethylphenylacetamidomethyl (OMPA) linkage to yield PAM resin which ensures improved stability to prolonged treatment with trifluoroacetic acid (TFA). A BOC-Val-OCH2-PAM-resin (0.78 mmol/g, 0.12863 g) was transferred to the reaction vessel of an ABI Peptide Synthesizer, model 430A (available from ABI, Foster City, CA). All subsequent amino acids starting from the carboxyl terminal to N-terminus were coupled in a stepwise manner using ABI's 0.1 mmol small scale rapid cycle protocol with capping (available from ABI, Foster City, CA, P/N 601781, version 1.40). Protected amino acids were coupled using preformed symmetric anhydride chemistry except for asparagine, glutamine, arginine and histidine which were double coupled using N-N'-dicyclohexylcarbodiimide (DCC)/1-hydroxybenzotriazole (HOBT) chemistry. In the first coupling, protected amino acids were coupled using acetic anhydride dissolved in dimethylformamide (DMF). The acetic anhydride of an individual amino acid was formed in methylene chloride followed by solvent exchange to DMF before transferring to the reaction vessel of the peptide synthesizer. The second coupling of symmetric anhydride was also conducted in DMF. The N-amino group of all amino acids

used was protected by a t-butyloxycarbonyl (t-BOC) linkage. The side chain functional groups of various amino acids were protected by the following groups:

Thr, Ser-Bzl	(Benzyl)
Asp, Glu-OBz	(O-Benzyl)
His-pBom	(p-Benzyloxy methyl)
Arg-Tos	(Tosyl)
Cys-4MeBzl	(4-Methyl benzyl)
Tyr-2BrZ	(2-Bromobenzyloxy carbonyl)
Lys-2ClZ	(2-Chlorobenzyloxy carbonyl)

Prior to placing the resin in the reaction vessel of the synthesizer, the fully protected peptide-resin (0.34137g) was allowed to swell in methylene chloride (CH_2Cl_2) for five minutes. The t-BOC protecting group was then removed using 60% TFA/CH_2Cl_2 according to the manufacturer's protocol and the partially deprotected peptide-resin was then dried overnight under house vacuum at room temperature.

Partially deprotected peptide-resin then was treated with p-cresol (0.6 ml) and p-thiocresol (0.5 g) in anhydrous HF (9 ml) at 0°C for one and three quarters hours to cleave the peptide from the resin support. The HF/DMS and other volatiles were distilled off in vacuo at 0°C. The cleaved peptide and resin were washed three times with 16 ml aliquots of diethyl ether, and the cleaved peptide was extracted by washing three times each with 10 ml aliquots of 40% aqueous acetic acid and 16% aqueous acetic acid, respectively. The aqueous extracts were combined and washed three times with 16 ml aliquots of diethyl ether and then lyophilized to yield a crude peptide.

The crude peptide was analyzed for purity using reversed-phase high performance liquid chromatography on a C4, 4.6 x 30 mm column (Brownlee, available from ABI, Foster City, California), flow rate one ml/minute employing 0.1% aqueous TFA (A) and 100% acetonitrile (B) as the solvent system. The preferred solvent gradient employed for this peptide analysis started with 33% B solvent. The column was maintained at 33% B for one minute followed by an increase over 20 minutes using a linear gradient to 63% B and maintained for one minute. Finally, the column was brought back to 33% B over one minute. The presence of peptide in the effluent was monitored simultaneously at 226 nm and 280 nm. The composition of the purified peptide was determined by acid hydrolysis. After removal of the acid, the hydrosylate was analyzed on a Beckman 6300 amino acid analyzer.

If increased quantities of purified polypeptide were desired, semipreparative reversed phase high performance liquid chromatography was performed in a similar manner using a C4, 10 x 100 mm column (Vydac, The Separations Group, Hesperia, CA) using the same aqueous 0.1% TFA (A) and 100% acetonitrile (B) solvent system described above. The preferred solvent gradient for a semi-preparative run started with 30% B at 3 ml/minute for five minutes followed by an increase, over 20 minutes, using a linear gradient to 45% B. The concentration was maintained at 45% B for one minute and then returned to 30% B in one minute.

D. Synthesis of SPM 42 (SEQ ID. NO. 1)

The fully protected peptide-resin was assembled on a phenylacetamidomethyl (PAM) resin by stepwise solid phase synthesis (starting with the carboxyl terminal residue) according to the general procedure of Barany and Merrifield mentioned previously. The C-terminal amino acid valine (Val) was coupled to the solid support vial an oxymethylphenylacetamidomethyl (OMPA) linkage to yield PAM resin which ensures improved stability to prolonged treatment with trifluoroacetic acid (TFA). A BOC-Val-OCH2-PAM-resin (0.77 mmol/g, 0.3170 g) was transferred to the reaction vessel of an ABI Peptide Synthesizer, model 430A (available from ABI, Foster City, CA). All subsequent amino acids starting from the carboxyl terminal to N-terminus were coupled in a stepwise manner using ABI's standard 0.5 mmole scale HOBT/N-methylpyrrolidine (NMP) chemistry protocol with capping (available from ABI, Foster City, CA, P/N 400 674, version 1.40). Protected amino acids were coupled using preformed symmetric anhydride chemistry except for asparagine, glutamine, arginine and histidine which were double coupled using N-N'-dicyclohexylcarbodiimide (DCC)/1-hydroxybenzotriazole (HOBT) chemistry. In the first coupling, protected amino acids were coupled using acetic anhydride dissolved in dimethyl-formamide (DMF). The acetic anhydride of an individual amino acid was formed in methylene chloride followed by solvent exchange to DMF before transferring to the reaction vessel of the peptide synthesizer. The second coupling of symmetric anhydride was also conducted in DMF. The N-amino group of all amino acids used was protected by a t-butyloxycarbonyl (t-BOC) linkage. The side chain functional groups of various amino acids were protected by the following groups:

Thr, Ser-Bzl	(Benzyl)
Asp, Glu-OBzl	(O-Benzyl)

His-pBom	(p-Benzyloxy methyl)
Arg-Tos	(Tosyl)
Cys-4MeBzl	(4-Methyl benzyl)
Tyr-2BrZ	(2-Bromobenzyloxy carbonyl)
Lys-2ClZ	(2-Chlorobenzyloxy carbonyl)
Asp-OBzl	(O-Benzyl)
Asp-OChxl	(O-cyclohexyl)
Glu-OBzl	(O-Benzyl)
Glu-OChxl	(O-cyclohexyl)

Prior to placing the resin in the reaction vessel of the synthesizer, the fully protected peptide-resin (0.46g) was allowed to swell in methylene chloride (CH_2Cl_2) for five minutes. The t-BOC protecting group then was removed using 60% TFA/CH_2Cl_2 according to the manufacturer's protocol and the partially deprotected peptide-resin was then dried overnight under house vacuum at room temperature.

Partially deprotected peptide-resin then was treated with dimethyl sulfide (DMS, 1 ml), p-cresol (1 ml) and p-thiocresol (0.2 g) in anhydrous HF (10 ml) at 0°C for two hours to cleave the peptide from the resin support. The HF/DMS and other volatiles were distilled off in vacuo at 0°C. The cleaved peptide and resin were washed three times with 15 ml aliquots of diethyl ether, and the cleaved peptide was extracted by washing three times each with 10 ml aliquots of 40% aqueous acetic acid and 16% aqueous acetic acid, respectively. The aqueous extracts were combined and washed three times with 15 ml aliquots of diethyl ether and then lyophilized to yield a crude peptide.

The crude peptide was analyzed for purity using reversed-phase high performance liquid chromatography on a Clg, 4.6 x 30 mm column (available from Vydac, The Separations Group, Hesperia, CA), flow rate one ml/minute employing 0.1% aqueous TFA (A) and 100% acetonitrile (B) as the solvent system. The preferred solvent gradient employed for this peptide analysis started with 25% B solvent. The column was maintained at 25% B for five minutes followed by an increase over 20 minutes using a linear gradient to 45% B and maintained for one minute. Finally, the column was brought back to 25% B over one minute. The presence of peptide in the effluent was monitored simultaneously at 225 nm and 280 nm. The composition of the purified peptide was determined by acid hydrolysis. After removal of the acid, the hydrosylate was analyzed on a Beckman 6300 amino acid analyzer.

If increased quantities of purified polypeptide were desired, semipreparative reversed phase high performance liquid chromatography was performed in a similar manner using a C18, 10 x 100 mm column (available from Vydac, The Separations Group, Hesperia, CA) using the same aqueous 0.1% TFA (A) and 100% acetonitrile (B) solvent system described above. The preferred solvent gradient for a semi-preparative run started with 27% B at 3 ml/minute for five minutes followed by an increase, over 20 minutes, using a linear gradient to 40% B. The concentration was maintained at 40% B for one minute and then returned to 27% B in one minute.

Example 2

Preparation of Capture PhaseWith Peptides

Peptides prepared as described in Example 1D (SEQ. ID.NO. 1) Example 1A (SEQ. ID.NO. 2), Example 1B (SEQ. ID.NO. 3) and Example 1C (SEQ. ID. NO. 4) were individually coated on polystyrene beads as the solid phase for use in subsequent assays to capture antibodies against HEV, as follows. Ground polystyrene beads (available from Abbott Laboratories, Abbott Park, IL) were washed with distilled water or propanol. Then, the beads were incubated at 56°C (range: room temperature to 60°C) for 2 hours rotating, shaking, or in another dynamic mode with a 0.5 to 20 µg/ml solution of peptide in a phosphate buffered saline (PBS) solution. After the incubation, the beads were washed in PBS. The beads then were blocked for 0.5 to 2 hour(s) with 5% BSA in PBS, overcoated with 5% sucrose in PBS for 15 to 60 minutes, and then dried.

Example 3

Preparation of Capture Phase With Recombinant Antigens

The four HEV recombinant antigens M 4-2, M 3-2, B 4-2 and B 3-2 (corresponding to SEQ. ID. NO.1, SEQ. ID . NO. 4. SEQ. ID. NO. 2 and SEQ. ID. NO. 3, respectively) were cloned and expressed in E. coli as fusion proteins with glutathione-S-transferase (GST) and supplied by Genelabs Technologies, Inc., Redwood City, CA. These recombinant antigens were individually coated on polystyrene beads as the solid phase for use in subsequent assays as a capture of antibodies against HEV, as follows. Ground polystyrene beads (available from Abbott Laboratories, Abbott Park, IL) were washed with distilled water or propanol. Then, the beads were incubated at 40°C (range: room temperature to 60°C) for 2 hours rotating, shaking, or in another dynamic mode with a 0.5 to 20 µg/ml solution of antigen in a phosphate buffered saline (PBS) solution. After the

incubation, the beads were washed in PBS. The beads then were blocked for 0.5 to 2 hour(s) with 5% bovine serum albumin (BSA) in PBS, overcoated with 5% sucrose in PBS for 15 to 60 minutes, and then dried.

Example 4

Detection of Anti-HEV IgG Antibody by ELISA

A. ELISA using HEV synthetic peptides. Test samples of serum were diluted 1:300 in specimen diluent comprising Phosphate buffered saline (PBS)/Tris/EDTA, pH7.8 containing 20% goat serum, 10% fetal calf serum, 1% bovine serum albumin, 0.2% Triton® X-100, 0.1% sodium azide. Then, 200 ul of the diluted sample was incubated with a solid phase (polystyrene bead) coated with synthetic peptides sp42 (SEQ. ID. NOS. 1 or 2) or sp33 (SEQ. ID. NOS. 3 or 4) prepared as described in Example 2 in a reaction tray for 2 hours at 40°C/static or room temperature/shaking. After incubation, the bead was thoroughly washed with distilled water, and then incubated for 60 minutes at 40°C/static or room temperature/shaking with goat anti-human HRPO (available from Kirkegaard and Perry, Gaithersburg, MD) diluted in Tris buffered saline containing 10% goat serum, 10% fetal calf serum, 0.15% Triton® X-100. The beads were thoroughly washed before adding 300 ul of OPD substrate reagent, freshly prepared by adding OPD tablets (available from Abbott Laboratories, Abbott Park, IL 60064) to 5 mls of OPD diluent per tablet (Abbott Laboratories, Abbott Park, IL, 60064). The bead and OPD substrate reagent were allowed to incubate at ambient temperature for 30 minutes before 1.0 ml of 1 N sufuric acid was added to stop the color generating reaction. The amount of color generation was determined by measuring the absorbance of the substrate at 492 nm within two hours after addition of the sulfuric acid.

B. ELISA using HEV recombinant proteins. An ELISA was performed as desribed in Example 4A, except that solid phases coated with recombinant proteins prepared as described in Example 3 were used in place of the solid phases having synthetic proteins coated thereon.

Results

This assay was applied to a panel of serum samples which had been obtained from 386 volunteer blood donors in Southeastern Wisconsin (a region considered as non-endemic for HEV infection). The samples were tested using solid phase capture reagents employing either HEV recombinant antigens corresponding to the amino acid sequences of HEV synthetic peptides SEQ. ID. NOS. 1, 2, 3 and 4. Eight of the 386 specimens tested were determined to be

reactive using assays employing the recombinant antigens. The eight specimens found reactive with the recombinant antigens also were found reactive with the method of the instant invention. The data obtained from the eight anti-HEV reactive specimens and their reactivities with the individual capture reagents is shown in TABLE 1. A positive symbol (+) indicates reactivity and a negative symbol (-) indicates non-reactivity.

TABLE 1

Reactivities of Eight Anti-HEV Reactive Wisconsin Specimens With Individual Synthetic and Recombinant Antigens

	Recombinant Proteins				Synthetic Peptides			
Specimen ID. NO.	B 4-2	B 3-2	M 4-2	M 3-2	SPB 33	SPB 42	SPM 33	SPM 42
60	-	-	-	+	-	+	+	+
96	+	+	-	-	+	-	+	-
174	+	+	-	-	+	-	+	-
199	-	+	-	-	+	+	+	+
242	+	+	+	-	+	-	+	-
265	+	+	+	-	+	+	+	+
354	+	+	-	-	+	-	-	+
353	+	+	+	-	+	-	+	+

The method also was applied to sera samples from 982 individuals from different global populations. The percentage of specimens from various regions which were repeatedly reactive for one or more recombinant antigens ranged from 2.3 to 16.0%. Approximately one half of the reactive specimens also were reactive with synthetic peptides, thus providing supportive evidence for their true reactivity. While evidence for confirmed anti-HEV antibody detection is low (1.1-3.0%) in the U.S., Germany, Japan and New Zealand, the confirmation in Mexico (7.0%) and Thailand (7.6%) was high. The data obtained from this study is shown in TABLE 2.

TABLE 2

Global Reactivities to Synthetic and Recombinant Antigens

Country	Total No. Samples Tested	Reaction with one or more Recombinant Proteins: No. Reactive	Recombinant Proteins: % Reactive	Synthetic Proteins: No. Reactive	Synthetic Proteins: % Reactive
Germany	151	11	7.3	3	2.2
Japan	100	8	8.0	3	3.0
Mexico	66	6	9.1	5	7.6
New Zealand	89	3	3.4	1	1.1
Thailand	100	15	15.0	7	7.0
United States:					
Alaska	90	4	4.4	1	1.1
Wisconsin	386	9	2.3	8	2.1

In another study, using the above method, the reactivities of HEV recombinant antigens and synthetic peptides were compared for 151 specimens obtained from an HEV outbreak in Somalia It was determined that 122 of 125 specimens reactive with recombinant antigens also were reactive with synthetic peptides, indicating an agreement of approximately 98%.

In a larger study, the above method was used to compare the reactivities of HEV recombinant antigens and synthetic peptides from 862 volunteer blood donors in Berlin, Germany. Thirteen of the specimens (1.51%) were reactive with one or more of the recombinant antigens and 10 (1.2%) were reactive with one or more of the synthetic peptides.

Example 7

Blocking Assay

Peptides prepared as described in Example 1D (SEQ. ID.NO. 1), Example 1A (SEQ. ID.NO. 2), Example 1B (SEQ. ID.NO. 3), Example 1C (SEQ. ID. NO. 4) were individually coated on polystyrene beads as the solid phase capture as described in Example 4A.

Polystyrene beads coated with synthetic peptides prepared as described in Examples 1A, 1B, 1C, 1D and 2 were used as solid phase antigens. Serum samples were diluted in a specimen diluent containing a blocking reagent (either recombinant antigen B 4-2 or M 3-2)in order to specifically inhibit antibodies

directed against HEV eptitopes from binding to the solid phase, or in a control reagent (recombinant HCV antigen) which did not block anti-HEV from binding to the solid phase. The recombinant HCV antigen was the same as that described in G. J. Dawson et al., J. Clin. Micro. 29: 1479-1486 (1990). The specimen diluent contained 25 to 500 µg/ml of the blocking or control reagent. Specimens whose absorbance values were reduced by more than 50% by the blocking reagent were considered reactive.

Samples were diluted (1:300) in specimen diluent containing blocking or control regent and incubated for 1 hour at room temperature to 40 C. 200ul of the diluted sample was then incubated with a bead coated with the corresponding synthetic peptide, and the ELISA was performed as described in Example 4A. Specimens whose absorbance values were reduced by more than 50% by the blocking reagent were considered reactive.

The above method was applied to the eight samples from the Southeastern Wisconsin donors which were found to be reactive with the recombinant antigens and synthetic peptides in Example 1. The binding of anti-HEV antibody, which previously bound a solid phase coated with various synthetic peptides of the instant invention, could be specifically inhibited by diluting the sample in diluent containing a recombinant antigen corresponding to the synthetic peptide coated on the solid phase. See G. J. Dawson et al., J.Virol. Methods. 38: 175-186 (1992), which is hereby incorporated by reference

Example 8

Reactivity of a CDC Panel

Eight specimens, the Center for Disease Control obtained from various ET-NANBH, were tested for anti-HEV (IgG and IgM) using methods and materials similar to those used in Example 5 and Example 6. A specimen from Burma was negative by all assays. Four specimens were reactive for IgG class antibodies to all four recombinant antigens. Seven of eight specimens were reactive with the B 4-2 epitope and six of these were confirmed with SPB 33 (SEQ.ID. NO. 3). Another Burmese specimen was reactive only with B 4-2. Specimens reactive with both B 4-2 and SPB 33 assays were also found to be reactive with at least two additional HEV recombinant antigens. In comparison, by the IgM specific assay, six of seven acute-phase sera reacted with B 4-2, five of seven with B 3-2 and M 4-2 and three of seven with M 3-2. See G. J. Dawson et al (1992) supra.

Example 9

Cynomolgus Macaques Study

A study to determine the immunoreactivity of sera obtained from experimentally infected cynomolgus macaques was conducted with both recombinant HEV proteins and synthetic peptides. Following their inoculation, all three animals produced antibodies reactive with one or more of the recombinant antigens or synthetic peptides. Some specimens were reactive with both recombinant antigens and synthetic peptides, while others were reactive for one or the other. See G. J. Dawson et al (1992) supra.

Example 10

Cross-Reactivity Study

Test sample obtained from patients and chimpanzees with antibodies against a variety of viruses and interfering globulins were tested by both anti-HEV IgG and IgM procedures as detailed in Example 4. The high specificity of the test procedures is demonstrated by the lack of cross-reactivity observed among specimens from various disease categories, as shown in Table 3.

TABLE 3

Cross-Reactivity Panel

		ANTI-HEV	
Disease Category	No. tested	IgG	IgM
Acute hepatitis A	34	3	3
Convalescent hepatitis A	5	0	0
Acute hepatitis B	10	1	1
Convalescent hepatitis B	10	0	0
Anti-HBc Reactive	10	0	1
Acute NANB	19	0	0
Rheumatoid Factor	20	2	0
ANA reactive	14	0	0
Acute rubella	5	0	0
Convalescent Norwalk vlrus	4	0	0

Example 11

Endpoint Dilution Studies

Six seropositive serum specimens were serially diluted in normal human serum devoid of anti-HEV and assayed by either the recombinant peptide prepred in GST or the synthetic peptide for B3-2 and B4-2 according to the procedures outlined in Example 4. The results of the study are presented in the following Table 4.

TABLE 4

Reciprocol Endpoint Dilutions

	B 3-2		B 4-2	
Sample No.	GST	sp	GST	sp
PC 1	4	0	8	4
PC 32	18	13	0	0
PC42	0	0	18	22
S43	7	7	8	16
S44	4	25	9	15
S 46	7	4	9	20

The data indicate that when a specimen was reactive with B 4-2, endpoints were higher when the synthetic peptide (sp) was used, as compared to the corresponding recombinant protein (GST). However, of the five specimens reactive with the B 3-2 sequence, three were more reactive with the recombinant protein (GST), compared to the synthetic peptide (sp).

It is contemplated that the assay of the invention can be optimized even further by varying assay conditions and/or incubation times, using various combinations of antigen or antibody capture or probe reagents, and other methods, reagents and conditions known to those skilled in the art. All these variations are contemplated to be within the scope of this invention. Also, it is well within the scope of the present invention that manual methods or automated analyzers can be used or adapted to the assay of the present invention. Therefore, the present invention is meant to be limited only by the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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PAUL, DEBORAH
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(ii) TITLE OF INVENTION: CONFIRMATORY ASSAY AND REAGENTS FOR HEPATITIS E VIRUS

(iii) NUMBER OF SEQUENCES: 4

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(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Ala Asn Gln Pro Gly His Leu Ala Pro Leu Gly Glu Ile Arg Pro Ser
1 5 10 15

Ala Pro Pro Leu Pro Pro Val Ala Asp Leu Pro Gln Pro Gly Leu Arg Arg
20 25 30

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Ala Asn Pro Pro Asp His Ser Ala Pro Leu Gly Val Thr Arg Pro Ser
1 5 10 15

Ala Pro Pro Leu Pro His Val Val Asp Leu Pro Gln Leu Gly Pro Arg Arg
20 25 30

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Thr Leu Asp Tyr Pro Ala Arg Ala His Thr Phe Asp Asp Phe Cys Pro
1 5 10 15

Glu Cys Arg Pro Leu Gly Leu Gln Gly Cys Ala Phe Gln Ser Thr Val
20 25 30

Ala Glu Leu Gln Arg Leu Lys Met Lys Val
35 40

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 42 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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Thr Phe Asp Tyr Pro Gly Arg Ala His Thr Phe Asp Asp Phe Cys Pro
1               5                  10                  15

Glu Cys Arg Ala Leu Gly Leu Gln Gly Cys Ala Phe Gln Ser Thr Val
            20                  25                  30

 Ala Glu Leu Gln Arg Leu Lys Val Lys Val
         35                  40
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WHAT IS CLAIMED IS:

1. A assay for confirming the presence of anti-HEV antibody in a test sample,.said method comprising:

(a) contacting the test sample with at least one synthetic peptide which specifically binds anti-HEV antibody, for a time and under conditions sufficient to form peptide/antibody complexes;

(b) contacting said peptide/antibody complexes with an indicator reagent which comprises a signal generating compound attached to an anti-human antibody, and incubating for a time and under conditions sufficient for peptide/antibody/antibody complexes to form; and

(c) detecting a measurable signal to indicate the presence of anti-HEV in the test sample.

2. The assay of claim 1 wherein said peptide of step (a) is attached to a solid phase prior to performing step (a) and wherein said solid phase is selected from the group consisting of microparticles, polystyrene beads and wells of a reaction tray.

3. The assay of claim 1 wherein steps (a) and (b) are performed simulateneously.

4. The method of claim 1 wherein said synthetic peptide is selected from the group consisting of SEQ. ID. NO. 1, SEQ. ID. NO. 2, SEQ. ID. NO. 3 and SEQ. ID. NO. 4.

5. The assay of claim 1 wherein said signal generating compound of said indicator reagent is selected from the group consisting of an enzyme, a luminescent compound, a radioactive element, a visual label and a chemiluminescent compound.

6. The assay of claim 5 wherein said enzyme is selected from the group consisting of horseradish peroxidase, beta-galactosidase and alkaline phosphatase.

7. A synthetic peptide selected from the group consisting of SEQ. ID. NO. 1, SEQ. ID. NO. 2, SEQ. ID. NO. 3 and SEQ. ID. NO. 4.

8. An assay kit for confirming the presence of human anti-HEV antibody in a test sample, comprising a container containing at least one synthetic peptide specific for anti-HEV antibody attached to a solid phase, and wherein said synthetic peptide comprises at least one peptide selected from the group consisting of SEQ. ID. NO. 1, SEQ. ID. NO. 2, SEQ. ID. NO. 3 and SEQ. ID. NO. 4.

9. The kit of claim 8 wherein said solid phase is selected from the group consisting of microparticles, polystyrene beads and wells of a reaction tray.

10. The kit of claim 9 further comprises a container containing an indicator reagent comprising an anti-human antibody attached to a signal generating compound, wherein said signal generating compound is selected from the group consisting of an enzyme, a luminescent compound, aradioactive element, a visual label and a chemiluminescent compound.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/07280

A. CLASSIFICATION OF SUBJECT MATTER
IPC(5) :C12Q 1/70; C07K 7/00
US CL :435/5; 530/324
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/5; 530/324; 436/518

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Journal of Virological Methods, Volume 38, issued 1992, G.J. Dawson et al., "Solid-phase enzyme-linked immunosorbent assay for hepatitis E virus IgG and IgM antibodies utilizing recombinant antigens and synthetic peptides," pages 175-186, see entire document.	1-10
Y	US, A, 5,106,726 (WANG) 21 April 1992, col. 5, lines 29-54; col. 23, line 54-col. 24, line 10.	1-10
Y	Journal of Virology, Volume 65, No. 11, issued November 1991, P. O. Yarbough et al., "Hepatitis E Virus: Identification of Type-Common Epitopes," pages 5790-5797, especially Figure 2.	1-10

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
12 OCTOBER 1994

Date of mailing of the international search report
20 OCT 1994

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/07280

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proceedings of the National Academy of Sciences USA, Volume 89, issued May 1992, M. Kaur et al., Human linear B-cell epitopes encoded by the hepatitis E virus include determinants in the RNA-dependent RNA polymerase, pages 3855-3858, especially page 3856.	1-10